The Effects of Environmental Enrichment on the Autism-like Behaviors of the BTBR Mouse Strain

an Undergraduate Honors Research Thesis

Presented in partial fulfillment of the requirements for graduation with Honors Research Distinction in the undergraduate colleges of The Ohio State University

by

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April 2019

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Abstract

BTBR *T+ Itpr3tf/J* (BTBR) mice are an Autism Spectrum Disorder (ASD)-like model and are characterized by similar behavioral and psychological symptoms as those presenting in patients with ASD. Such symptoms include difficulty in communication, impaired sociability, and increased engagement in repetitive stereotypic behaviors. Behavioral therapy is a common treatment for human ASD, but such non-pharmacological interventions remain to be explored in murine models. Here, we explored the effects of environmental enrichment (EE)—a housing condition consisting of larger space, running wheels, toys, etc. designed to stimulate complex social and cognitive engagement—in alleviating the symptoms of ASD. Juvenile BTBR mice were placed in standard or EE housing for 17 weeks while undergoing several behavioral and metabolic assessments. Following EE-exposure, male BTBR mice displayed reduced adiposity, increased lean mass, improved glycemic control, and decreased circulating leptin. Regarding behavior, EE-BTBR males displayed decreased anxiety and improved social affiliation but no changes in repetitive behaviors or social memory. Additionally, a gene expression profile of males showed an EE-induced upregulation of brain-derived neurotrophic factor (BDNF) and its receptor TrkB in several brain regions. Contrarily, female BTBR responded less readily to EE, displaying only modest adipose remodeling and no changes in glycemic control, circulating leptin, anxiety, sociability, or repetitive behaviors. These results suggest that EE improves metabolic and some behavioral phenotypes in a sexually-dimorphic manner in BTBR mice.

Introduction

Autism Spectrum Disorder (ASD) is a common neurodevelopmental disorder that is characterized by the following criteria: difficulty in social communication and interactions,

restricted interests, and engagement in repetitive stereotypic behaviors [1]. Comorbidity with other disorders such as depression, anxiety, and obesity is common in children diagnosed with ASD [2]. According to the most recent estimates from the Centers for Disease Control and Prevention's (CDC) Autism and Developmental Monitoring Network, approximately 1 in 59 children have been identified with ASD in the United States [3]. Additionally, the prevalence of this disorder is varied by sex with males presenting four times more likely than females to be diagnosed [3]. The exact causes of ASD remain unknown, but numerous studies suggest that its onset is influenced by a combination of genes as well as environment. ASD is considered a public health issue and year after year its prevalence continues to rise.

Patients with ASD are comprised of a highly heterogeneous group, sharing only a common behavioral phenotype. In order to explore the heterogeneity of this disorder, several mouse models paralleling the varying mutations that are present in human ASD have been identified. Such models include valproic acid (VPA)-induced, Shank3B, and BTBR *T+ Itpr3tf/j* (BTBR). After discovering an association between prenatal exposure to VPA and an increase risk of developing ASD, several investigators developed VPA-induced ASD-like behavior in rodents in order to explore treatments to ameliorate symptoms [4]. The Shank3B mouse model has been studied due to the comparatively high prevalence of SHANK3 mutations found in human ASD. These mice display deficits in excitatory neurotransmission and synaptic plasticity as well aberrant autistic-like behaviors, as noted by social impairment and increased repetitive behaviors [5].

Unlike the chemically induced VPA model and the genetic Shank3B model, the BTBR mouse strain serves to represent idiopathic autism. Multiple genetic, neuroanatomical, and molecular irregularities comprise the BTBR background [6]. Among these include

downregulated brain-derived neurotrophic factor (BDNF), an absence of a corpus callosum, and an excitatory/inhibitory (E/I) neurotransmission imbalance, most notably with glutamate [7]. Studies using conventional neuroimaging technology suggest that impaired neuronal activation and cognitive function demonstrated in BTBR mice may be a reflection of reductions in cerebral blood flow and cerebral oxygen metabolism [8].

Ultimately, BTBR mice were chosen for our experiment for their strong behavioral phenotype. These mice display face validity to all three diagnostic core symptoms of autism: impaired sociability, communication deficits, and restricted repetitive behaviors [9]. It is also important to note metabolic deficiencies observed in BTBR mice; the strain was originally bred for research studying insulin-resistance, diabetes-induced nephropathy, and phenyloketonuria [6]. High-fat diet administration in BTBR mice has also been shown to aggravate autism-related behaviors such as increased cognitive rigidity and diminished social novelty preference [10], highlighting a correlation between the metabolic and behavioral phenotype.

Presently, there are no pharmacotherapies approved by the US Food and Drug Administration to effectively treat all of the core symptoms of ASD. The antipsychotics risperidone and aripiprazole are the only FDA-approved pharmacological treatments, but they are limited to treatment of associated irritability symptoms [11]. Consequently, many drug therapies are being investigated in rodents, most notably targeting the E/I imbalance. Previous studies have shown that the administration of gaboxadol, a GABA-A agonist, results in a reduction of excessive repetitive behavior in BTBR mice, suggesting that GABA agonists work by targeting impairments in inhibition [5]. Reduction of excitatory neurotransmission through inhibition of mGluR receptors by negative allosteric modulators has also been shown to rescue social deficits and repetitive self-grooming behavior in BTBR mice [12]. Many novel

pharmacological targets are being tested in mouse models in order to discover replicable and therapeutic results that may then be pursued in clinical trials.

Although psychopharmacological therapies are not yet effective in humans, treatment through interventional therapies at a young age have been successful in alleviating challenges and symptoms associated with ASD. Early Intensive Behavioral Intervention (EIBI) has been effective in ameliorating social challenges in autistic children when administered at a young age [13]. A meta-analysis reviewing programs utilizing Cognitive Behavioral Therapy (CBT) suggests that CBT treatments may be effective in producing moderate changes in anxiety levels in children with ASD through management of thoughts and emotions [14]. Additionally, individualized Sensory Enrichment Therapy delivered online is promising for treating a wide array of ASD symptoms including learning, memory, anxiety, attention span, communication, social skills, and mood [15]. Successful human intervention treatment paves way for the development of similar treatment models in mice in order to further decipher the underlying mechanisms of ASD.

Previous work has shown that environmental enrichment (EE), a model of eustress, leads to anti-obesity, anti-cancer, and anxiolytic phenotypes [16]. EE is designed to stimulate complex social, cognitive, and somatosensory engagement and the housing condition is composed of a larger space, mazes, running wheels, and toys [17]. EE decreases adiposity, increases energy expenditure, resists diet induced obesity, and causes cancer remission and inhibition in C57Bl/6 mice, all through the activation of the hypothalamic-sympathoneural- adipocyte (HSA)-axis [18-20]. In response to EE, hypothalamic *Bdnf* is induced as an effector immediate early gene which consequently increases the circulating BDNF protein. The upregulation of this protein results in the activation of sympathetic innervation of white adipose tissue (WAT), an element of HSA-

activation [16]. HSA-activation is associated with metabolic changes and is comprised of many components including an increase in hypothalamic BDNF, suppression of leptin expression and release, and a reduction in WAT mass [16]. It has also been shown that EE modulates immune functions in models of cancer and autoimmune disease via hypothalamic BDNF [16, 21]. Many studies have demonstrated that EE modulates an anti-cancer and anti-obesity phenotype. However, the potential of EE to modulate sociability and anxiolytic effects, particularly in the BTBR mouse strain, still remains to be explored.

The main purpose of this study was to investigate the effects of environmental enrichment as an intervention treatment for the ASD-like symptoms of the BTBR mouse model. A second aim was to assess the extent to which EE could improve the metabolic profile of BTBR mice. It was hypothesized that EE would ameliorate anxiety, low sociability, repetitive behaviors, and metabolic deficiencies characterized in this ASD-like murine model.

Methods

EE Protocol

4-5 week old male and female BTBR *T+ Itpr3tf*/J (Jackson Laboratory #002282) were randomized to live in EE or standard laboratory conditions. Control mice were group housed (3-5 mice) in standard laboratory environment cages (30.5 cm x 17 cm x 15 cm). EE mice were group housed (3-5 mice) in large cages (63 cm x 49 cm x 44 cm) supplemented with running wheels, igloos, toys, tunnels, a maze, and nesting material. Mice had *ad libitium* access to food (normal chow diet, 11% fat, caloric density 3.4 kcal/g, Teklad) and water. Mice were housed in temperature (22-23ºC) and humidity (30-70%) controlled rooms under a 12:12 light:dark cycle.

All animal experiments were in accordance with the regulations of the Ohio State University Institutional Animal Care and Use Committee.

Glucose Tolerance Test (GTT)

Mice were injected intraperitoneally with glucose solution (1.0 mg glucose per kg body weight) after a 16 h overnight fast. Blood was obtained from the tail at 15, 30, 60, 90, and 120 min after glucose injection. Blood glucose concentrations were measured with a portable glucose meter (Bayer Contour Next).

Open Field (OF) Test

Mice were individually placed into the center of an open square arena (60cm x 60cm, enclosed by walls of 48 cm). Each mouse was allowed to explore the arena for 10 min, during which time activity was recorded and analyzed via TopScan (Clever Sys Inc) software. The specific parameters analyzed included total distance travelled and periphery and center distances in relation to total distance. Between each trial, the arena was cleaned with 30% ethanol to remove odor cues.

Three-Chamber Sociability (TCS) Test

Mice were placed in an apparatus consisting of three connected plexiglass chambers (custom, 18 cm x 41 cm x 20 cm) with removable dividers between each chamber. Each mouse was individually placed in the center chamber for 5 minutes of habituation. In the first test, an unfamiliar mouse was placed in either the right or left chamber in a small wire cage while the opposing wire cage remained empty. The dividers were lifted to allow the test mouse to move

freely about all three chambers for a 10-minute period. The placement of the unfamiliar mouse in the right vs. left chamber was systematically alternated between each trial. The wire cage allowed for nose contacts but restricted the stranger mouse from initiating social contact and limited aggressive interactions between the two mice.

A second 10-minute test was performed using the mouse from the first test (now denoted as a familiar mouse) and a novel unfamiliar mouse. All trials were video recorded and a blinded experimenter analyzed for the time spent in each chamber and number of chamber entries. An entry was defined by 4 paws in the chamber. All cages were cleaned with opticide between each animal trial in order to eliminate odor effects.

Grooming Test

Mice were individually placed in a small empty cage (28 cm x 16 cm x 12 cm) and were video recorded for 10 min. A blinded experimenter scored self-grooming as indicated by paw licking, nose/face/head wash, body grooming, leg licking, and/or tail/genital grooming. The total number of grooming bouts and the average length of each bout for each mouse were measured.

Marble Burying Test

Mice were individually placed into cages (30.5 cm x 17 cm x 15 cm) with evenly spaced glass marbles arranged in a three-by-four grid on the surface of clean aspen bedding (5 cm in depth). Cages were covered with plexiglass lids to prevent mouse escape. Mouse burying activity was video recorded for 30 min with an overhead camera. A blinded experimenter scored the latency to bury as well as the number of marbles buried. The marbles were washed with mild

detergent and water between each trial.

Quantitative Real-Time-PCR

Amygdala, hippocampus, and hypothalamus were dissected at sacrifice. Tissues were flash frozen on dry ice and stored at -80°C until further analysis. Following sonication, RNA was isolated using the QIAGEN RNeasy Mini kit with RNase-free DNase treatment. cDNA was reverse transcribed using Taqman Reverse Transcription Reagents (Applied Biosystems). qRT-PCR was completed on a ABI PRISM 7000 Sequence Detection System using Power SYBR Green (Applied Biosystems) PCR Master Mix. Primers are available upon request.

Serum Harvest and Analysis

Trunk blood was collected at euthanasia. Serum was allowed to clot on ice for at least 30 min before centrifugation at 10,000 rpm for 10 min at 4°C. Serum was collected and stored at -20°C until further analysis. DuoSet ELISA kits were used to assay serum leptin (R&D Systems #DY498) and adiponectin (R&D Systems #DY1119). Triglycerides were measured using a colorimetric assay kit (Cayman Chemical #10010303)

Body Composition by EchoMRI

EchoMRI was utilized to measure body composition of fat, lean, free water, and total water masses in live mice without anesthesia. EchoMRI was performed with an EchoMRI 3-in-1 Analyzer at the Small Animal Imaging Core of The Dorothy M. Davis Heart & Lung Research Institute, The Ohio State University.

Statistical Analysis

Data are expressed as mean ± SEM. We used GraphPad Prism 7 software (GraphPad, La Jolla, CA) and SPSS Statistics v25.0.0.0 (IBM, Armonk, NY) to analyze the following: student's t test for body weight or food intake at single time points, adiposity, tissue weights, serum ELISAs, behavior, and quantitative RT-PCR data. Mixed analysis of variance was performed on time course measurements (body weight, GTT).

Tissue Collection

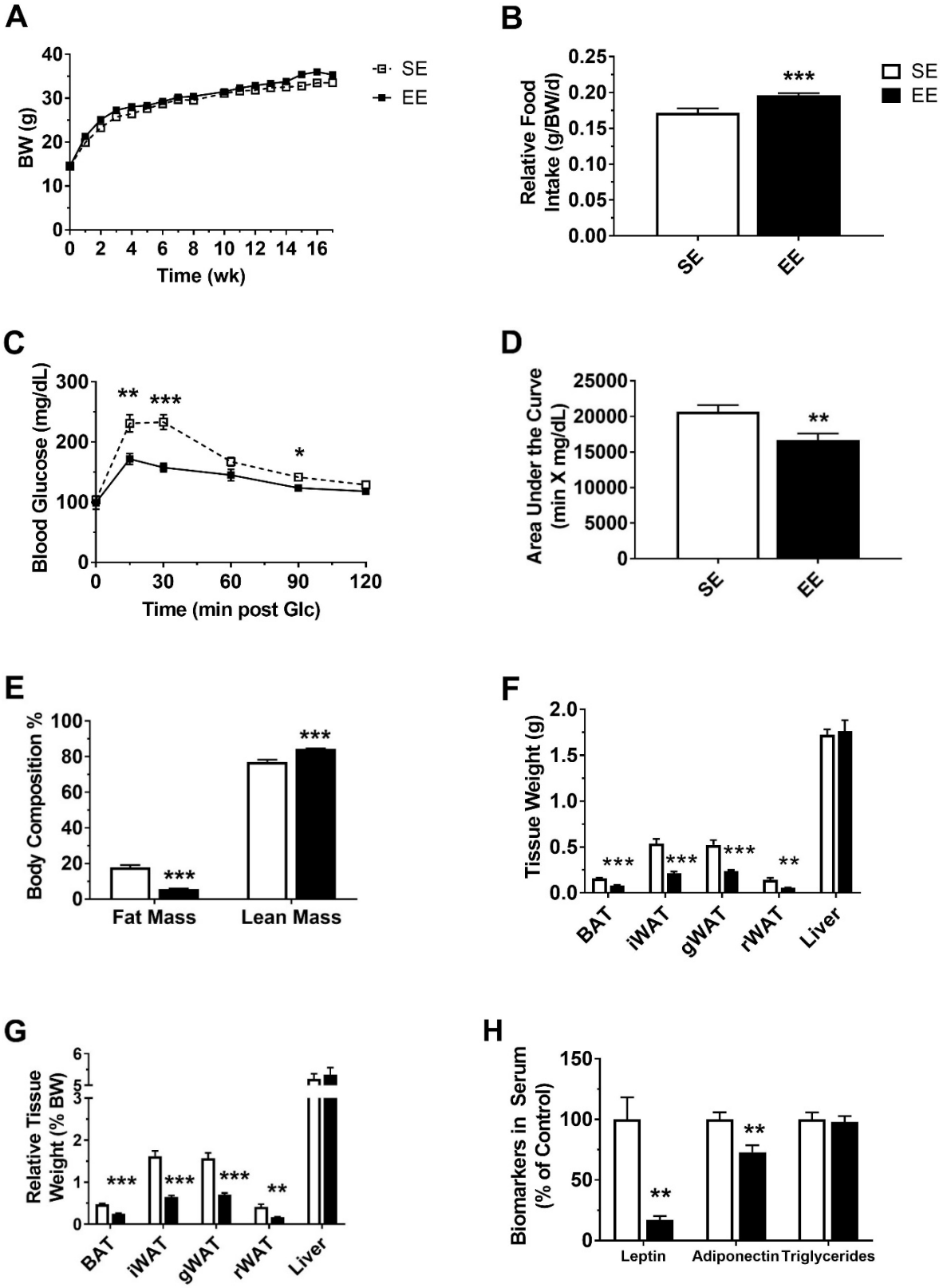
Following 17 weeks of housing conditions, mice were sacrificed. Mice were euthanized cage-wise beginning at 0930 h. Tissues were flash frozen on dry ice and stored at -80°C until further analysis.

Results

EE Induces Sex-differentiated Activation of the HSA axis

EE modulated metabolism of the BTBR mouse model in a sexually-dimorphic manner. The metabolic results of the male cohort are presented in Figure 1. Although male EE mice consumed a greater amount of food relative to body weight (Fig. 1B), there were no significant differences in body weight by the conclusion of the experiment (Fig. 1A). Previous results suggest that EE produces thermogenic effects and acts by elevating energy expenditure, which may account for these findings [18]. At 12 week EE, EchoMRI was used to measure *in vivo* body composition. It was found that EE significantly decreased fat mass while increasing lean mass (Fig. 1E). A GTT performed at 16 week EE further revealed a significant improvement in male glycemic control (Fig. 1C, D). A reduction of adiposity in EE was again confirmed at sacrifice,

as evidenced by decreased relative brown adipose tissue (BAT), inguinal white adipose tissue (iWAT), gonadal white adipose tissue (gWAT), and retroperitoneal white adipose tissue (rWAT) mass (Fig. 1F,G). The signature of the serum biomarkers often associated with EE in previous studies involving C57BL/6 mice was examined. In serum, male EE-BTBR mice displayed a significant reduction of leptin and adiponectin, with no change in triglycerides (Fig. 1H). Quantitative Real-Time-PCR revealed a significant upregulation of relative BDNF mRNA expression in the hypothalamus, which is consistent with an increased activation of the HSA-axis (Fig. 1I). No differences were seen in the amygdala or hippocampus. Lastly, there was a significant upregulation of expression of BDNF receptor trkB mRNA in the hypothalamus, amygdala, and hippocampus (Fig. 1J).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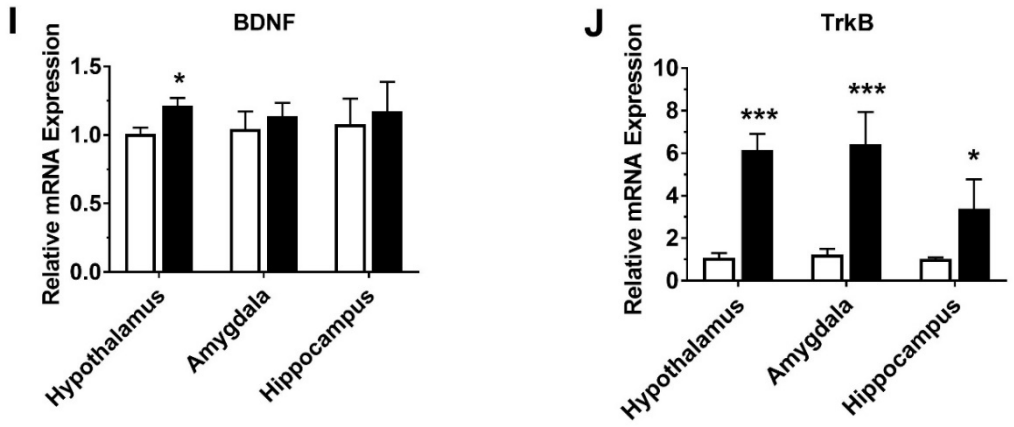
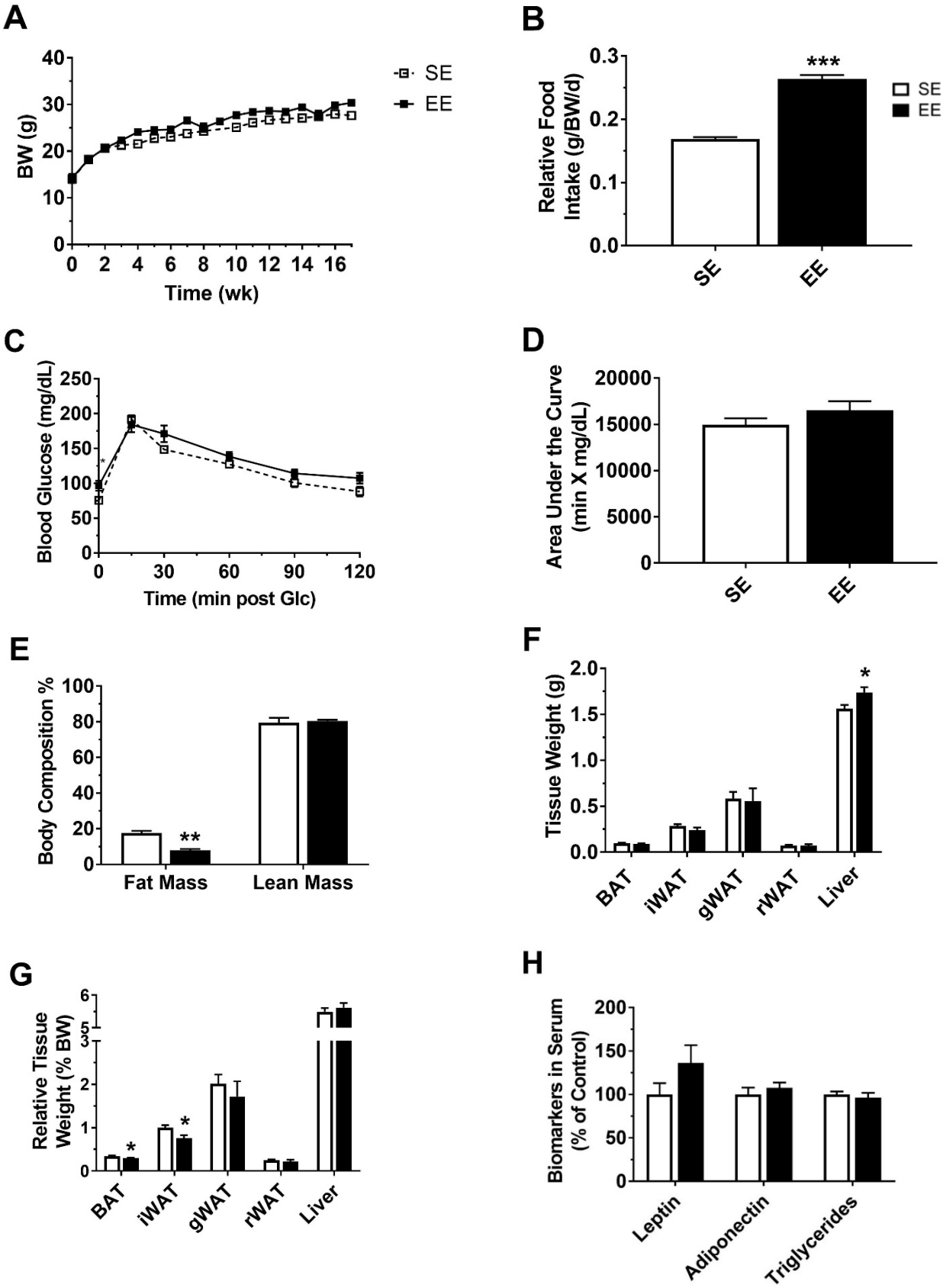


Figure 1. EE-related metabolic changes and gene expression profiling in male BTBR mice. (A) Body weight. (B) Relative food intake. (C) Glucose tolerance test, performed 16 weeks post housing. (D) Area under the curve (AUC) of (C). (E) echoMRI body composition assessment, performed at 12 weeks post housing. (F) Gross tissue mass at sacrifice. (G) Relative tissue mass at sacrifice. (H) Serum biomarkers at sacrifice. (I) Relative mRNA expression of Bdnf. (J) Relative mRNA expression of full-length TrkB. Data are means ± SEM. n=8 SE, n=9 EE. * * P<0.05, ** P<0.01, *** P<0.001.

Female mice exhibited a starkly different metabolic phenotype as presented in Figure 2. Similar to male mice, EE had no significant effects on the body weight (Fig. 2A), but significantly increased food consumption relative to body weight (Fig. 2B). While female mice showed a significant decrease in fat mass, there was no significant differences in lean mass as measured by echoMRI (Fig. 2E). Furthermore, EE had no significant effect on glycemic control of female mice (Fig. 2C, D). EE-induced female reductions in adiposity were more modest than those seen in male counterparts. EE resulted in a decreased relative BAT and iWAT mass (Fig. 2G). No significant differences in biomarkers of female mice were found (Fig. 2H). Due to the sexual dimorphism seen in between the male and female cohorts, relative mRNA expression of female mice was not analyzed.


A
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Relative Food Intake (g/BW/d)
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Area Under the Curve (min X mg/dL)
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Body Composition %
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Fat Mass
Lean Mass
F
Tissue Weight (g)
*
BAT
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gWAT
rWAT
Liver
G
Relative Tissue Weight (% BW)
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BAT
iWAT
gWAT
rWAT
Liver
H
Biomarkers in Serum (% of Control)
Leptin
Adiponectin
Triglycerides

Figure 2. EE-related metabolic changes and gene expression profiling in female BTBR mice. (A) Body weight. (B) Relative food intake. (C) Glucose tolerance test, performed 16 weeks post housing. (D) Area under the curve (AUC) of (C). (E) echoMRI body composition assessment, performed at 12 weeks post housing. (F) Gross tissue mass at sacrifice. (G) Relative tissue mass at sacrifice. (H) Serum biomarkers at sacrifice. Data are means ± SEM. n=10 SE, n=9 EE. * P<0.05, ** P<0.01, *** P<0.001.

Our data suggest that EE is modulating metabolism of the BTBR mouse model in a sex-specific manner. Key features of HSA axis activation, particularly the up-regulation of hypothalamic Bdnf, reduction of adiposity, and reduction of circulating leptin, were confirmed in male EE mice but not seen in their female counterparts.

EE Induces Anxiolytic Behavior

Over the course of the experiment, BTBR mice underwent a series of behavioral experiments. The OF test is based on the natural instinct of mice to want to explore a new environment, yet avoid an exposed area. An increase in relative time spent in the center of the arena is considered to reflect a reduction in anxiety. There was no significant difference in the total distance traveled by the male EE mice (Fig. 3A). However, male EE mice travelled a proportionally greater distance in the center of the arena (Fig. 3B) than the periphery (Fig. 3C), suggesting an anxiolytic effect. Conversely, female EE mice traveled a significantly greater total distance suggesting increased locomotion in this cohort (Fig. 3D). There were no significant differences in the distance traveled by female EE mice in the periphery and center relative to the total distance (Fig. 3E, F).

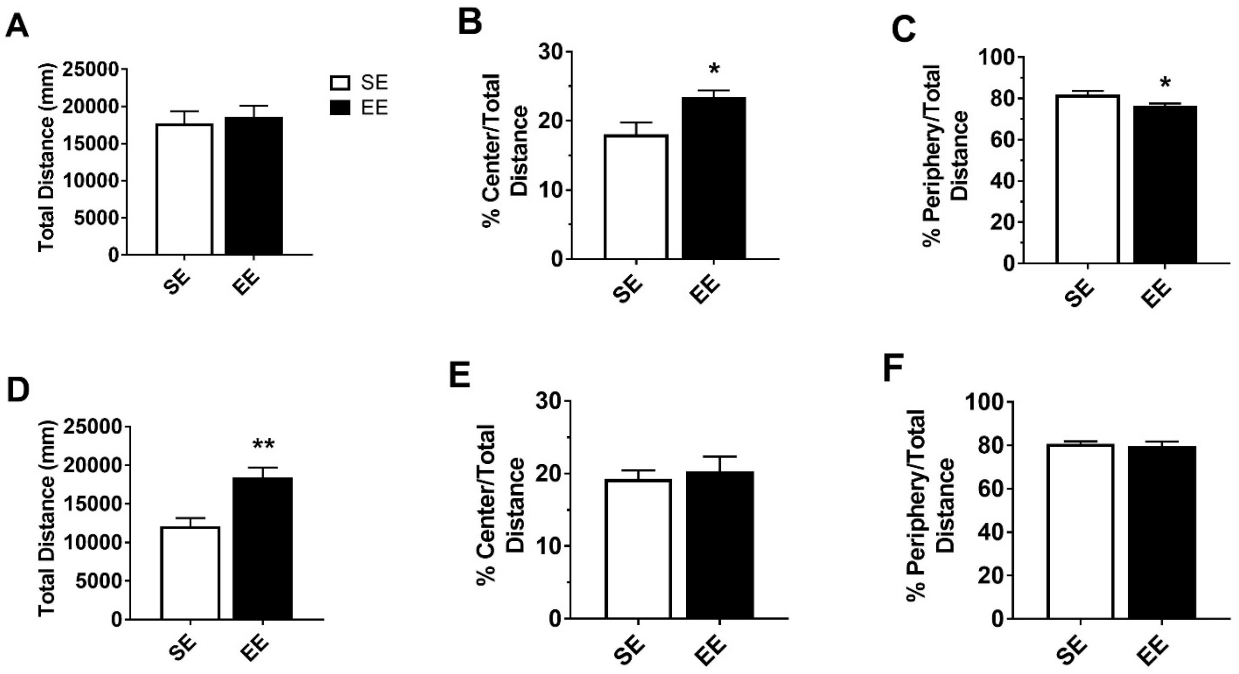

Figure 3. (A-C) Male Open field Test. (A) Total distance traveled. (B) Relative distance traveled in the center of the arena. (C) Relative distance traveled in the periphery of the arena. (D-F) Female Open field Test. (D) Total distance traveled. (E) Relative distance traveled in the center of the arena. (F) Relative distance traveled in the periphery of the arena.

Data are means ± SEM. n=9 male EE; n=8 male SE; n=9 female EE; n=10 female SE. * P<0.05, ** P<0.01, *** P<0.001.

EE's Effect on Repetitive Behaviors

Assessments of grooming and marble burying were performed in order to evaluate repetitive and OCD-like symptoms. BTBR mice have been shown to exhibit excessive self-grooming behavior [22]. A decrease in the number of total bouts as well as the length of those bouts would be suggestive of an attenuation of these symptoms. No significant effects were observed in the Grooming Test (Fig. 4). Digging has been indicated as a parallel to motor stereotypy and thus the MBT was conducted to analyze repetitive, compulsive-like behaviors. An increased number of marbles buried correlates to a greater severity of OCD-like symptoms [22]. No significant differences were found in MBT (Fig. 5). Our data suggest that EE does not

alleviate the repetitive and compulsive-like symptoms of BTBR mice.

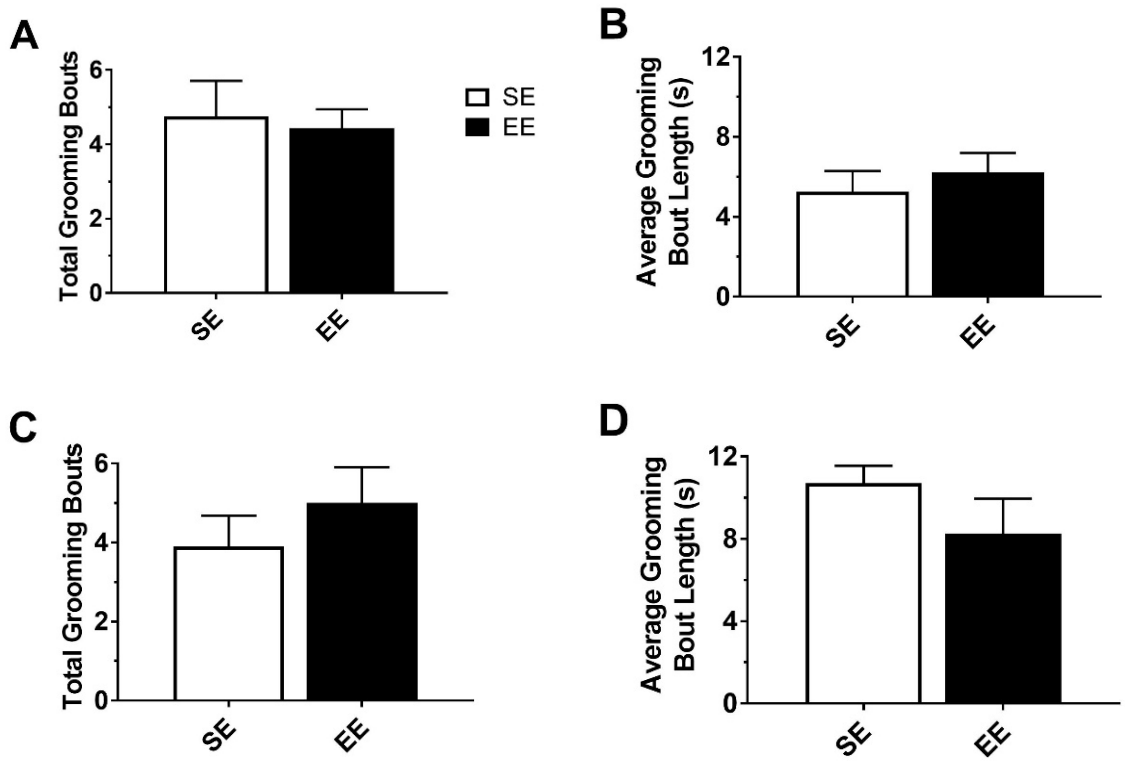

Figure 4. (A-B) Male Grooming Test. (A) Total number of grooming bouts. (B) Average length of each grooming bout. (C-D) Female Grooming Test. (C) Female total number of grooming bouts. (D) Average length of each grooming bout.

Data are means ± SEM. n=9 male EE; n=8 male SE; n=9 female EE; n=10 female SE. *P<0.05, ** P<0.01, *** P<0.001

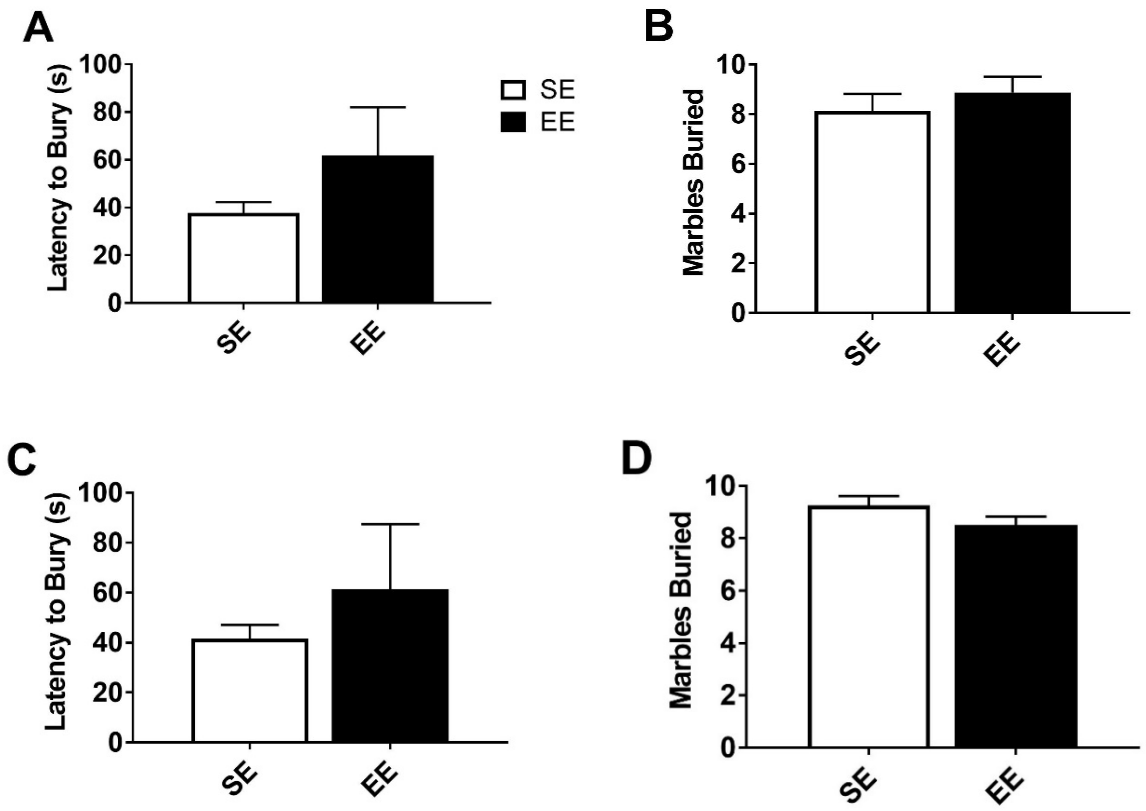

Figure 5. (A-B) Male Marble Burying Test. (A) Latency to bury first marble. (B) Total number of marbles buried. (C-D) Female Marble Burying Test (C) Latency to bury first marble (D) Total number of marbles buried.

Data are means ± SEM. n=9 male EE; n=8 male SE; n=9 female EE; n=10 female SE. * P<0.05, ** P<0.01, *** P<0.001

EE Effect on Social Behavior

Lastly, the TCS test was performed to assess how EE affects social behavior, particularly social affiliation and social memory. Mice were allowed to explore three chambers while interacting with social stimuli. In the first test phase, which is thought to assess social affiliation, mice were exposed to a novel confined peer and an opposing empty chamber. In the second phase test, thought to assess social memory, mice were exposed to both a novel and a familiar confined peer in opposing chambers. During the first social affiliation phase, male EE mice spent significantly more time in the chamber containing a social stimulus and significantly less time in the empty chamber when compared to male SE mice (Fig. 6A). During the second test phase, no

significant improvements in social memory were seen in male EE mice (Fig. 6B). Last but not least, no significant effects were found on either the social affiliation (Fig. 6C) or social memory (Fig. 6D) of female mice.

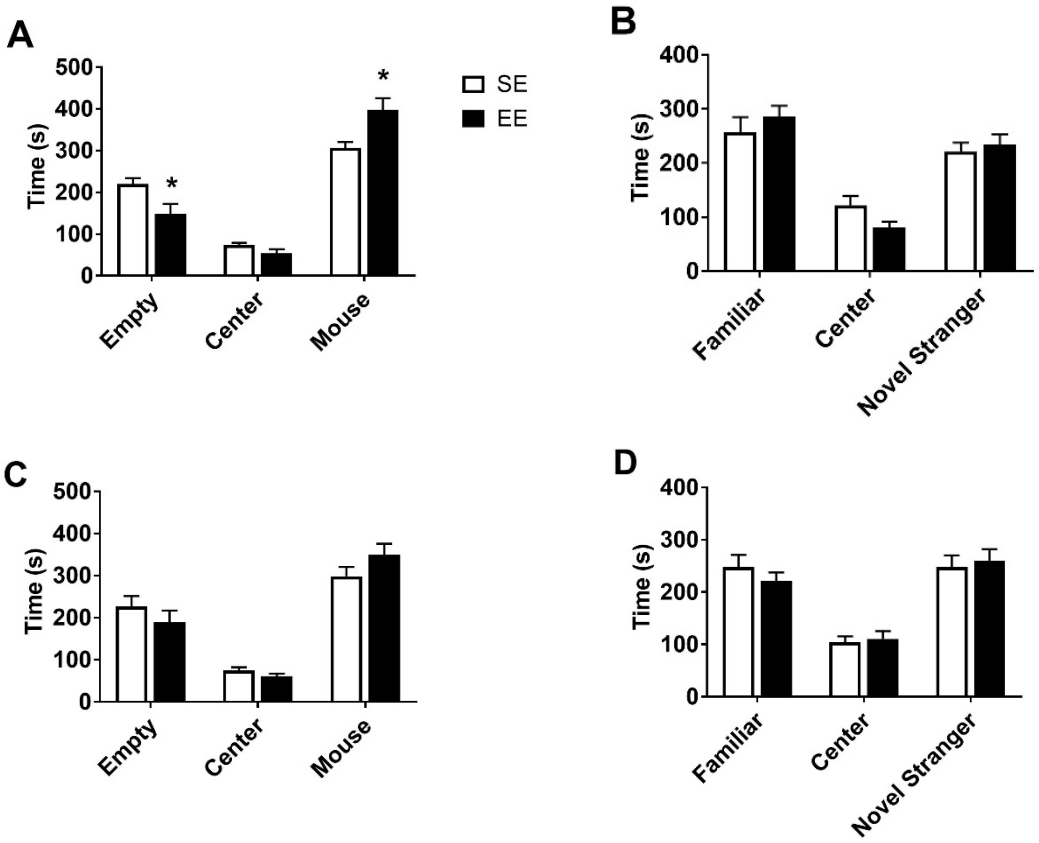

Figure 6. (A-B) Male Three Chamber Sociability Test. (A) First phase—Social Affiliation (B) Second phase--Social Memory (C-D) Female Three Chamber Sociability Test (C) First phase—Social Affiliation (D) Second phase—Social Memory. Data are means ± SEM. n=9 male EE; n=8 male SE; n=9 female EE; n=10 female SE. * P<0.05, ** P<0.01, *** P<0.001

Discussion

The BTBR mouse strain is a widely used model for ASD but to date, very few studies have examined the effects of EE on their behavioral deficits. A literature search reveals that publications combining the BTBR model and the EE paradigm are limited. With regard to the available research, one group has found that EE increases novel object recognition in the BTBR

strain [23]. Another group has shown EE to significantly reduce the amount of time BTBR mice are engaged in repetitive self-grooming behaviors [24]. Additionally, one study has demonstrated how pairing BTBR mice with unimpaired C57BL/6 mice, a form of social peer enrichment, mitigates the low sociability phenotype presented by the BTBR strain. In our investigation, we hypothesized that EE would attenuate ASD-like symptoms and the depressed metabolic phenotype of the BTBR model. Our findings suggest that EE exposure improves the metabolic and some behavioral phenotypes of the BTBR murine model in a sex-dependent manner.

HSA activation has been implicated as an important feature of EE treatment as presented in previous studies of C57BL/6 mice [16]. In this study, male EE mice exhibited reduced adiposity, decreased leptin levels, and improved glycemic control. Furthermore, results also showed an upregulation of BDNF and TrkB within the hypothalamus. This suggests that EE induces HSA axis activation and the subsequent metabolic improvement in male BTBR mice. On the other hand, female EE mice responded less readily to EE treatment. They displayed a more modest adiposity reduction and no significant changes in glycemic control, leptin levels, or serum biomarkers. The resulting data suggest that EE mediates activation of the HSA-axis in sexually dimorphic manner.

Hallmark behavioral symptoms of ASD include impairments in communication, atypical social interactions, and excessive repetitive behaviors [1]. Comorbid psychiatric conditions are common in children with ASD and include, but are not limited to ADHD, anxiety, and depression [25]. In order to validate EE as a model for improving BTBR social deficiencies, three autism-like characteristics of the BTBR mouse strain were assessed: anxiety, repetitive behaviors, and impaired sociability. OF test results suggest that EE treatment produces anxiolytic effects in male mice but no changes in anxiety levels were seen in female mice. No significant

changes in repetitive behavior in both male and female mice were observed in the Marble Burying and Grooming tests. TCS test results suggest that EE treatment induces increased social affiliation and exploratory behavior in male mice, but no changes in these behaviors were seen in females. Social memory did not improve in either the male or female cohorts. Ultimately, these data suggest that there is sex-dependent improvement in some behavioral deficits in BTBR mice following EE exposure.

It is also important to consider the connections between neuroanatomical differences and behavioral changes. Transcriptomic, proteomic, and histopathologic data suggest impaired neurogenesis and reduction in hippocampal protein levels of BDNF and TrkB in BTBR mouse brains [26, 27]. BDNF has been established as a key mediator in the mechanistic pathways of EE-induced immune and metabolic changes [16]. A large body of literature also indicates EE as increasing BDNF levels and thus neurogenesis, a pathway for behavioral treatment [28-30]. One such study showed how transplantation of mesenchymal stem cells in BTBR mice, resulting in increased hippocampal BDNF levels and neurogenesis, significantly improved their autism-like behavioral deficiencies [31]. BDNF has further been identified as critical link between environmental stimulation and neurogenesis as demonstrated by a complete abrogation of the effects of EE on hippocampal neural stem cell proliferation and survival in BDNF deficient mice [32]. Early social enrichment has led to increased BDNF levels in the hippocampus and prefrontal cortex of adult mice which has been implicated in shaping brain plasticity and regulating social development [33]. Furthermore, overexpression of hypothalamic BDNF has also been shown to reduce anxiety-like behaviors in middle age female C57BL/6 mice [20].

In this investigation, EE-related stimuli resulted in the upregulation of hypothalamic BDNF. However, further studies are required to explore the role of BDNF in mitigating

behavioral deficiencies as seen in BTBR mice. Overexpressing BDNF in the hypothalamus would allow researchers to see whether upregulating this protein could mimic the EE effects on metabolism, anxiety, and social affiliation that have been observed in male BTBR mice. Results also signified an upregulation of the TrkB receptor in the male hypothalamus, hippocampus, and amygdala. Given the link between EE, BDNF, and neurogenesis, future research should investigate the potential for increased neurogenesis in the EE-BTBR model. Additional studies are necessary to decipher upstream and downstream regulators and to elucidate ASD-relevant mechanisms. Our results indicate that TrkB and BDNF may be promising targets for future mechanistic work.

Some limitations of this study exist. Due to the early sexual dimorphism seen between the male and female cohorts, relative mRNA expressions of female mice were not analyzed. However, laboratory analysis of genetic expression profiling of *Bdnf* and TrkB is necessary to confirm any significant differences between the sexes.

Some may attribute the metabolic and behavioral changes seen in this study as simply a result of EE mice having more opportunity for exercise. One finding suggests that physical activity may be sufficient enough to enhance several aspects of hippocampal neurogenesis [34]. However, another study has demonstrated a significant additive upregulation in the gene expression of *Bdnf* following 4 week EE in comparison to voluntary running [19]. This suggests that EE treatment might be working through more holistic means by stimulating additional somatosensory and social pathways. In order to elucidate the mechanisms of neurogenesis and *Bdnf* in the BTBR mouse model, further studies utilizing the EE versus voluntary running paradigm must be conducted.

It is also necessary to consider replicability of results. When performing future studies, there should be a consistency in utilizing established standardized EE protocols [17]. Unlike a previous study that reported decreased stereotypic self-grooming behavior following EE exposure [24], our study did not find any changes in grooming. Both studies adhered to the same protocol for analyzing grooming behavior [35], but may have differed due to temporal differences or divergences in EE protocol.

Last but not least, our study found a sex-related difference in the metabolic and behavioral changes promoted by EE. As mentioned earlier, males are four times more likely than females to be diagnosed with ASD [3], indicating that there may be differing underlying causes. One study has found sex-related differences in the gut microbiota profile and behavioral phenotype of BTBR mice [36]. The connection between the gut-brain axes is currently being explored and it may be interesting to look at the effects of EE on the gut microbiome of the BTBR male and female mice. Research investigating the sex-related differences in human autism is understudied and inconsistent [37]. Utilizing mouse models allows researchers to more clearly understand observed sex-related differences.

In conclusion, this study presents initial findings of EE-induced positive changes in the metabolic and behavioral profile of male BTBR mice. Early intervention treatments are helpful for ameliorating ASD symptoms in children. Further studies examining the effects of enrichment in murine models will allow for researchers to elucidate the underlying mechanisms of ASD and develop more efficient therapies.

ACKNOWLEDGEMENTS

I would like to express gratitude toward Dr. Lei Cao for this learning opportunity and for fostering my growth as a scientist. I thank Nicholas Queen for technical training, technical assistance, and editorial assistance. I thank Amber Boardman for technical assistance. I additionally thank Jason Siu for technical training in the laboratory. I would like to acknowledge Seemaab Ali and Wei Huang for aide in tissue collection. I thank Dr. Zachary Weil, Julie Fitzgerald, and the Behavior Core at The Ohio State University for materials and consultation regarding the Three-Chamber Sociability Test. This work was supported by NIH grants AG041250, CA166590, CA178227, CA163640 to L.C. Additional support provided by P30NS104177.

REFERENCES

1. Association, A.P., *Diagnostic and statistal manual of mental disorders*. 5th ed. 2013, Arlington, VA: American Psychiatric Publishing.

2. Simonoff, E., et al., *Psychiatric Disorders in Children With Autism Spectrum Disorders: Prevalence, Comorbidity, and Associated Factors in a Population-Derived Sample.* Journal of the American Academy of Child & Adolescent Psychiatry, 2008. **47**(8): p. 921-929.

3. Baio, J., et al., *Prevalence of Autism Spectrum Disorder Among Children Aged 8 Years — Autism and Developmental Disabilities Monitoring Network, 11 Sites, United States, 2014.* MMWR. Surveillance Summaries, 2018. **67**(6): p. 1-23.

4. Ornoy, A., et al., *Prevention or Amelioration of Autism-Like Symptoms in Animal Models: Will it Bring Us Closer to Treating Human ASD?* International Journal of Molecular Sciences, 2019. **20**(5): p. 1074-1074.

5. Rhine, M.A., et al., *Hypothesis-driven investigations of diverse pharmacological targets in two mouse models of autism.* Autism Research, 2019. **12**(3): p. 401-421.

6. Meyza, K.Z. and D.C. Blanchard, *The BTBR mouse model of idiopathic autism-current view on mechanisms.*

7. Daimon, C.M., et al., *Hippocampal transcriptomic and proteomic alterations in the BTBR mouse model of autism spectrum disorder.* Frontiers in Physiology, 2015. **6**(NOV): p. 1-17.

8. Abookasis, D., et al., *Optically derived metabolic and hemodynamic parameters predict hippocampal neurogenesis in the BTBR mouse model of autism.* Journal of Biophotonics, 2018. **11**(3): p. e201600322-e201600322.

9. McFarlane, H.G., et al., *Autism-like behavioral phenotypes in BTBR T+tf/J mice.* Genes, Brain and Behavior, 2008. **7**(2): p. 152-163.

10. Zilkha, N., Y. Kuperman, and T. Kimchi, *High-fat diet exacerbates cognitive rigidity and social deficiency in the BTBR mouse model of autism.* Neuroscience, 2017. **345**: p. 142-154.

11. Kazdoba, T.M., et al., *Translational Mouse Models of Autism: Advancing Toward Pharmacological Therapeutics.* Current topics in behavioral neurosciences, 2016. **28**: p. 1-52.

12. Silverman, J.L., et al., *Repetitive self-grooming behavior in the BTBR mouse model of autism is blocked by the mGluR5 antagonist MPEP.* Neuropsychopharmacology, 2010. **35**(4): p. 976-989.

13. Waters, C.F., et al., *Sustainability of Early Intensive Behavioral Intervention for Children With Autism Spectrum Disorder in a Community Setting.* Behavior Modification, 2018: p. 014544551878646-014544551878646.

14. Perihan, C., et al., *Effects of Cognitive Behavioral Therapy for Reducing Anxiety in Children with High Functioning ASD: A Systematic Review and Meta-Analysis.* Journal of Autism and Developmental Disorders, 2019: p. 1-15.

15. Aronoff, E., R. Hillyer, and M. Leon, *Environmental Enrichment Therapy for Autism: Outcomes with Increased Access.* Neural Plasticity, 2016. **2016**: p. 23.

16. Cao, L. and M.J. During, *What Is the Brain-Cancer Connection?* Annual Review of Neuroscience, 2012. **35**: p. 331-345.

17. Slater, A.M. and L. Cao, *A Protocol for Housing Mice in an Enriched Environment.* Journal of Visualized Experiments : JoVE, 2015(100).

18. Cao, L., et al., *White to Brown Fat Phenotypic Switch Induced by Genetic and Environmental Activation of a Hypothalamic-Adipocyte Axis.* Cell Metabolism, 2011. **14**(3): p. 324-338.

19. Cao, L., et al., *Environmental and Genetic Activation of a Brain-Adipocyte BDNF/Leptin Axis Causes Cancer Remission and Inhibition.* Cell, 2010. **142**(1): p. 52-64.

20. McMurphy, T., et al., *Hypothalamic gene transfer of BDNF promotes healthy aging in mice.* Aging Cell, 2018.

21. Xiao, R., et al., *Enriched environment regulates thymocyte development and alleviates experimental autoimmune encephalomyelitis in mice.* Brain, Behavior, and Immunity, 2019. **75**(October 2018): p. 137-148.

22. Angoa-Pérez, M., et al., *Marble burying and nestlet shredding as tests of repetitive, compulsive-like behaviors in mice.* Journal of visualized experiments : JoVE, 2013(82): p. 50978-50978.

23. MacPherson, P., et al., *Impaired fear memory, altered object memory and modified hippocampal synaptic plasticity in split-brain mice.* Brain Research, 2008. **1210**: p. 179-188.

24. Reynolds, S., M. Urruela, and D.P. Devine, *Effects of environmental enrichment on repetitive behaviors in the BTBR T+tf/J mouse model of autism.* Autism Res, 2013. **6**(5): p. 337-43.

25. Tye, C., et al., *Characterizing the interplay between autism spectrum disorder and comorbid medical conditions: An integrative review.* Frontiers in Psychiatry, 2018. **9**: p. 751.

26. Cartocci, G., et al., *Reduced social interaction, behavioural flexibility and BDNF signalling in the BTBR T+tf/J strain, a mouse model of autism.* Behavioural Brain Research, 2012.

27. Stephenson, D.T., et al., *Histopathologic characterization of the BTBR mouse model of autistic-like behavior reveals selective changes in neurodevelopmental proteins and adult hippocampal neurogenesis.* Molecular Autism, 2011. **2**(1).

28. Rogers, J., T. Renoir, and A.J. Hannan, *Gene-environment interactions informing therapeutic approaches to cognitive and affective disorders.* Neuropharmacology, 2019. **145**: p. 37-48.

29. Fan, D., et al., *Enriched Environment Attenuates Surgery-Induced Impairment of Learning, Memory, and Neurogenesis Possibly by Preserving BDNF Expression.* Molecular Neurobiology, 2016. **53**(1): p. 344-354.

30. Jha, S., B. Dong, and K. Sakata, *Enriched environment treatment reverses depression-like behavior and restores reduced hippocampal neurogenesis and protein levels of brain-*

derived neurotrophic factor in mice lacking its expression through promoter IV. Translational Psychiatry, 2011. **1**(9): p. e40-11.

31. Segal-Gavish, H., et al., *Mesenchymal Stem Cell Transplantation Promotes Neurogenesis and Ameliorates Autism Related Behaviors in BTBR Mice.* Autism Research, 2016. **9**(1): p. 17-32.
32. Rossi, C., et al., *Brain-derived neurotrophic factor (BDNF) is required for the enhancement of hippocampal neurogenesis following environmental enrichment.* European Journal of Neuroscience, 2006. **24**(7): p. 1850-1856.
33. Branchi, I., et al., *Early Social Enrichment Shapes Social Behavior and Nerve Growth Factor and Brain-Derived Neurotrophic Factor Levels in the Adult Mouse Brain.* Biological Psychiatry, 2006.
34. Henriette, V.P., K. Gerd, and G. Fred, *Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus.* Nature Neuroscience, 1999. **2**(3): p. 266-270.
35. Kalueff, A.V., et al., *Analyzing grooming microstructure in neurobehavioral experiments.* Nature Protocols, 2007. **2**(10): p. 2538-2544.
36. Coretti, L., et al., *Sex-related alterations of gut microbiota composition in the BTBR mouse model of autism spectrum disorder.* Scientific Reports, 2017. **7**: p. 45356-45356.
37. Green, R.M., et al., *Women and Autism Spectrum Disorder: Diagnosis and Implications for Treatment of Adolescents and Adults.* Current Psychiatry Reports, 2019. **21**(4): p. 22-22.